Chapter 1

General Introduction
&
Review of Literature

Medicine Nobel 2023 awarded to Katalin Karikó, Drew Weissman for their work in mRNA vaccines

The Nobel Prizes for 2023 in Medicine or Physiology has been awarded to Katalin Karikó and Drew Weissman

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1.1. General Introduction

Genetic disorders rooted in abnormalities within an individual's DNA encompass a broad spectrum of conditions that can impact various aspects of health, leading to a wide array of health issues. Genetic disorders arise from alterations in an individual's DNA, either inherited from parents or occurring spontaneously [1-3]. These alterations, known as mutations, can affect the structure or function of proteins, resulting in a variety of disease conditions. The disorders can manifest in childhood or later in life, affecting the nervous, musculoskeletal, cardiovascular, metabolic systems etc. Managing these genetic disorders presents unique challenges as many of these conditions are rare, making diagnosis and treatment complex [4-6]. The hereditary nature of these disorders also poses challenges in terms of genetic counselling. Additionally, the genetic basis often means there is no cure, and treatments focus on alleviating symptoms and improving the quality of life only.

Historically, two main categories of medications that the US FDA has approved are small molecule medicines and proteins [7]. Traditional pharmacological techniques depend on the capacity of small molecule medicines to target the active areas of proteins in order to block or change their function. However, knowing that only 1.5% of the human genome codes for proteins is important and that only 10-14% of proteins have active binding sites that can be "druggable" by small compounds [8-10]. As a result, there aren't many "druggable" targets for small molecule treatments. While small-molecule drugs inhibit target proteins through competitive binding, protein-based medications (like antibodies) can bind to multiple targets with great selectivity [11-13]. However, the proteins usefulness as majority of disease targets is severely constrained by their size and stability, and neither protein-specific nor small-molecule medicines are able to specifically target all disease-relevant proteins or genes. Candidate genes for other dominantly inherited diseases are also not obvious targets for small-molecule or antibody drugs, despite the fact that antibody-based and conformation-correcting therapeutics are being developed to improve the removal or spread of protein species that are implicated in causing some instances of such genetic diseases. Additionally, recessively inherited genes have produced a modest number of candidate proteins for creating traditional small molecule medications. Thus, the problem is that only a portion of targets that have been identified as being associated with human diseases may be directly addressed by small-molecule or protein-based therapies.

1.2. Antisense Oligonucleotides (ASOs): A Promising Frontier in RNA Therapeutics

In the age of functional genomics, more focus has been placed on effective techniques for silencing genes, as the sequencing of the human genome and the genomes of various model species revealed a large number of genes with unknown functions. In the ever-evolving landscape of RNA therapeutics (Figure 1.1), Antisense Oligonucleotides (ASOs) have emerged as powerful agents with the potential to revolutionize the treatment of various genetic disorders [14-15]. ASOs represent a class of synthetic macromolecules designed to selectively bind to target RNA sequences, modulating gene expression and providing a targeted approach to addressing the root causes of genetic diseases [16-19]. ASOs have the potential to treat any disease, including viral infections, cancer, and inflammatory disorders, that is brought on by the expression of a harmful gene. Typical composition of the ASOs is 15-20 nucleotides that complement the mRNA of their target. Target validation, functional genomics, and therapeutic applications can all benefit from the use of antisense compounds, which are useful tools for sequence-specific inhibition of target gene expression. One of the significant advantages of ASOs is their ability to provide targeted therapeutics. This precision arises from the customizable nature of these oligonucleotides, allowing researchers to design sequences that specifically match the target RNA thus minimizing off-target effects and enhances the therapeutic potential of Antisense Oligonucleotides (ASOs).

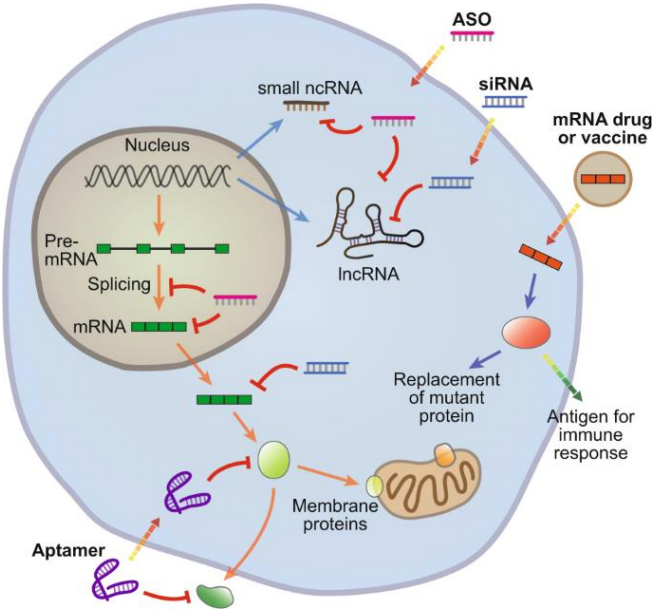

Figure 1.1: Pictorial representation of the various RNA therapeutics including ASOs, mRNA vaccines, siRNAs, aptamers and their varying mechanism of actions. Figure reproduced from [20] by Springer Nature.

1.2.1. Antisense Therapeutics: ASO optimization, challenges, next-generation ASOs, Rise and Future Scenario of the ASOs

When compared to competing technologies for gene functionalization and target validation, ASOs offer a high probability of success, broad applicability, direct usage of sequence information, rapid development at low costs, and high specificity. For instance, the commonly used method of creating knock-out animals to learn more about the function of genes *in vivo* is labor-intensive, costly, time-consuming, and, in many circumstances non-informative because of mortality of the embryo during development. In these situations, antisense technologies present a compelling substitute for precisely inhibiting a target gene's expression [21]. Another advantage is that ASOs were successfully used to study a secondary role of E-cadherin when given later in development stage wherein mouse E-cadherin embryos were dead at an early stage of embryogenesis because they are unable to form the blastocoele. Though theoretically quite elegant, antisense techniques have proven difficult to use in actual applications.

Long RNA molecules can create intricate secondary and tertiary structures. The number of active ASOs is known to differ between targets, and on an average, only one in eight ASOs is believed to bind effectively and to a given target mRNA [22]. Consequently, determining the mRNA's accessible target locations is the first step in a successful antisense method. Long RNA molecules in computer-based structure models are unlikely to accurately reflect the RNA structure inside a real cell, and these models are currently only partially helpful for creating efficient ASOs. For this reason, many other approaches have been developed [23]. One may easily test the antisense efficiency of several ASOs; however, more sophisticated techniques are acknowledged for a methodical tuning of the antisense effect. It has been demonstrated that utilizing random or semirandom ASO libraries in conjunction with RNase H and primer extension can provide a complete image of the accessible sites [24-25]. By breaking down the template DNA, a non-random variant of this technique was created that produced target-specific ASOs [26]. Screening a large number of particular ASOs against the transcript in the presence of RNase H and measuring the amount of cleavage generated by each ASOs is a fairly easy and basic technique that yields equivalent information on the structure of the target RNA [27]. Creating a DNA array to map an RNA for ASOs hybridization sites is the most advanced method that has been documented so far [28]. Because mRNA architectures in biological systems are expected to differ from the structure of produced

RNA molecules in vitro and because RNA-binding proteins shield specific target locations inside cells, screening the efficacy of ASOs in cell extracts [29] or in cell culture may be helpful [30-31].

It is not advisable to use ASOs with four consecutive Guanosine residues because they may generate G-quartets through the creation of Hoogsteen base pairs, which could lower the amount of ASOs that is available and cause unfavourable side effects [22]. This issue may be solved by using modified guanosines, such as 7-deazaguanosine, which is unable to generate Hoogsteen base pairs [32]. Since CpG motifs are known to elicit immunological responses in mammalian systems, they should also be avoided in *in vivo* investigations involving ASOs [22]. Also, CG dinucleotide is more commonly found in the DNA of viruses and bacteria than in the human genome, and it may serve as an immune system marker for infection. Coley Pharmaceuticals uses CG-containing ASOs as immune stimulants to treat infectious illnesses, cancer, and asthma [33].

ASOs hold the potential to unlock new possibilities in the realm of personalized medicine, paving the way for innovative treatments tailored to individual genetic profiles. However, in order to prevent considerable homology with other mRNAs, a database search for each ASO sequence is a crucial stage in the construction of an antisense molecule. Furthermore, to demonstrate that any effect seen is the result of a particular antisense knockdown of the target mRNA, control tests must be done extremely carefully. For antisense research, a variety of control ASOs have been employed, including random ASOs, scrambled ASOs with the same nucleotide composition as the ASO in random order, sense ASOs, ASOs with the inverted sequence, and mismatch ASOs which only differ from the ASOs in a small number of locations [34-35]. Additionally, issues like *in vivo* stability, cellular absorption, and toxicity have frequently impeded the use of ASO agents and must be resolved for their successful implementation. Furthermore, data from investigations relating to ASOs have been misinterpreted due to non-antisense effects of ASOs [36]. Therefore, another requirement for the successful use of ASOs is the presence of suitable controls to demonstrate that any observed effect is caused by a particular antisense suppression of gene expression. Despite their great potential, there are still obstacles to be resolved, including effective delivery to target tissues, the possibility of off-target effects and the requirement for long-lasting therapeutic benefits. Ongoing research focuses on addressing these challenges and optimizing the ASO designs to enhance their clinical efficacy.

1.3. The Evolution of Antisense Oligonucleotides (ASOs): A Historical Review

The history of ASOs reflects a trajectory from conceptualization to clinical realization and the journey of the ASOs as a therapeutic modality is marked by decades of scientific exploration and technological advancements reflecting the persistence of researchers in unravelling the potential of manipulating gene expression for therapeutic purposes.

1.3.1. Early Concept and Discovery (1970s-1980s)

The foundation of ASOs development can be traced back to the pioneering work of Paul Zamecnik and Mary Stephenson in the 1970s (Figure 1.2). They hypothesized that synthetic nucleotides could be designed to bind to complementary RNA sequences and interfere with protein synthesis. The idea of an antisense nucleic acid was first introduced when single-stranded nucleic acids were employed to prevent RNA translation in a cell-free system in the year 1977. In 1978, Zamecnik and Stephenson published their groundbreaking research work describing the inhibition of Rous Sarcoma Virus (RSV) replication using synthetic Antisense Oligonucleotides (ASOs), a landmark moment in the history of ASOs [37].

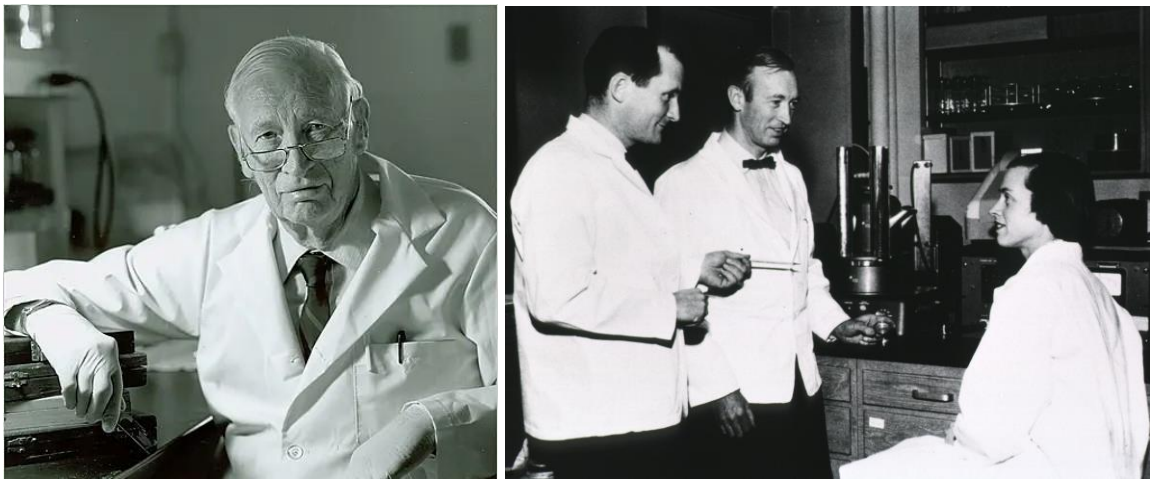

Figure 1.2: Left picture: Dr. Paul C. Zamecnik. **Right picture: S**tanding, right pose Dr. Mahlon B. Hoagland, **S**tanding, left pose Dr. Paul C. Zamecnik and Seated, left profile Dr. Mary L. Stephenson. Figure reproduced from NIH National Library of Medicine, Digital Collections [38].

Since then, target validation and therapeutic applications have benefited greatly from the development of antisense technology. Although there was tremendous progress in understanding the application of such ASOs, at that time, little advancement was achieved in this domain over the course of time, mostly due to the lack of synthetic procedures for producing a sizable quantity of oligonucleotides (ON).

1.3.2. Development of Antisense Technology (1980s-1990s)

Ever since the first documented use of exogenously administered synthetic antisense oligodeoxynucleotides (ODNs) to decrease Rous Sarcoma Virus expression in tissue culture, there has been a lot of support as well as debate surrounding the use of antisense oligonucleotides (ASOs). The 1980-1990s witnessed the refinement of antisense technology. Automated DNA synthesis first appeared in the 1980s, which sped up the development of antisense technology as a novel approach to genetic therapy and a tool for studying gene function [39]. Followed by this, researchers explored various aspects, including optimizing oligonucleotide design, enhancing stability, improving delivery methods and including challenges such as rapid degradation by cellular nucleases and inefficient delivery limited early applications. These antisense investigations have made extensive use of nucleotides that have undergone chemical modification.

Ribonucleotides can generally be modified in three different locations (Figure 1.3) *i.e.,* the sugar ring (at the 2' position of the ribose) [40-41], phosphate backbone [42-43] and analogues with foreign bases. In order to improve base-pairing and hence stabilize the duplex between ASOs and their target mRNAs, a number of heterocyclic modifications have also been developed [44]. However, little is known about the potential of these ASOs as antisense agents and their potential hazardous side-effects because only a tiny portion of them have been studied *in-vivo*. Despite these hurdles, the potential of ASOs in modulating gene expression captured the imagination of the scientific community.

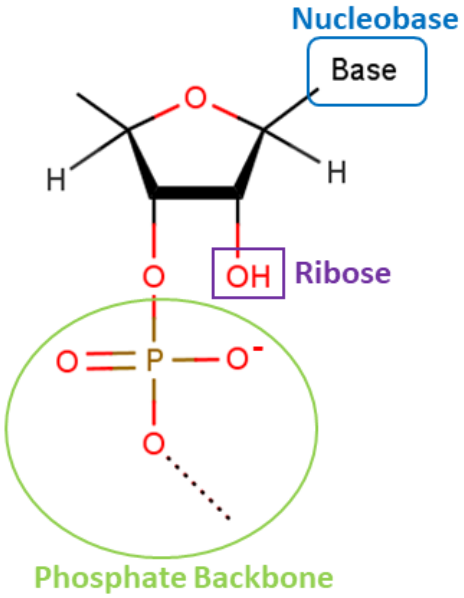

Figure 1.3: Probable sites for chemical modifications in RNAs. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

1.3.2.1. First-generation Antisense Oligonucleotides (ASOs) (beyond 1990s)

Development of innovative chemical modifications to stabilize ASOs against nucleolytic degradation and improve their target affinity has shown significant success in recent years. The stability of ASOs is one of the main obstacles for antisense methods since unmodified ODNs are quickly broken down by nucleases in biological fluids. The most prominent first-generation DNA analogue (Figure 1.4), which is the most well-known and frequently utilized ASO to date is the phosphothioate (PS) oligodeoxynucleotide (ODN).

In this class of ASOs, sulphur is substituted for one of the nonbridging oxygen atoms in the phosphodiester bond. The first PS-DNA ODNs were created in 1969 by Eckstein and colleagues [45], and Matsukura and colleagues utilized them as ASOs to stop HIV replication in 1987 [46]. The main goal of adding PS-DNA connections to ODNs was to increase their nuclease resistance. Compared to unmodified ODNs with half-life of about ~1 hour, PS-DNA ODNs have a half-life of about ~9-10 hours in human serum [47-48]. PS-DNA ODNs have favourable pharmacokinetic characteristics, generate regular Watson-Crick base pairs, activate RNase H, carry negative charges for cell transport, and are nuclease resistant. [49].

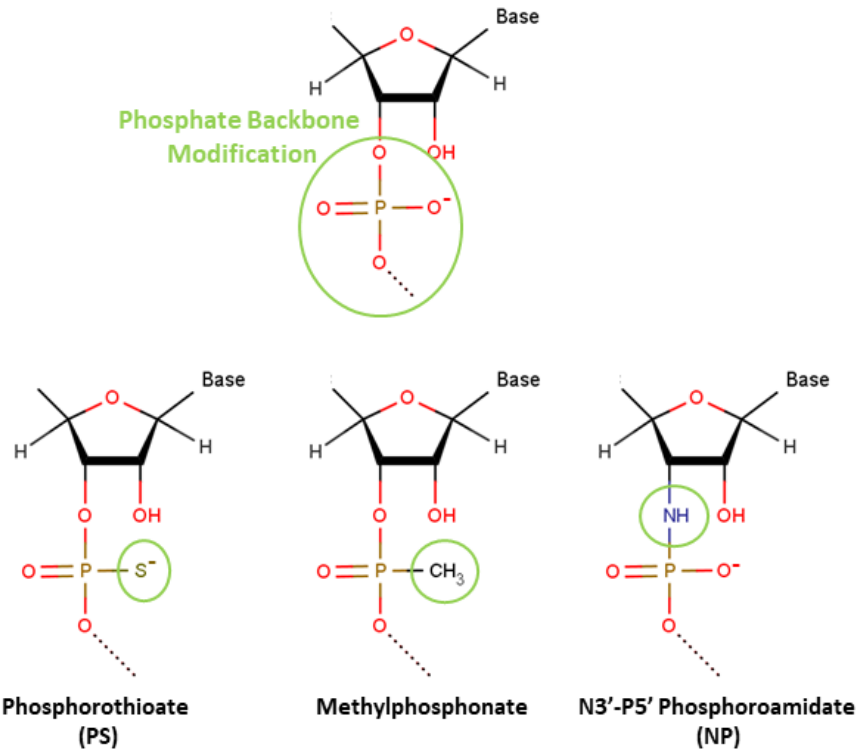

Figure 1.4: Noteworthy First-generation nucleic acid analogues. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

Despite the fact that these PS-DNA ODNs have several advantages, they also have some disadvantages. The primary drawback of PS-DNA ODNs is that they attach to specific proteins, especially those that interact with polyanions, including heparin-binding proteins [50-53]. The transitory activation of the complement cascade following PS-DNA ODN treatment of primates resulted in substantial acute toxicity, which in some cases resulted in cardiovascular collapse and death. Although it was unclear as to why this non-specific contact occurs, but they were toxic to cells. Additionally, the injection of PS-DNA ODNs changed the coagulation cascade. However, the lower doses of PS-DNA ODNs utilized in clinical studies in people were typically well tolerated. Additionally, the PS-DNA ODNs apparently unfavourable ability to bind with specific proteins turned out to be advantageous for the pharmacokinetic profile. They are shielded from filtration by their attachment to plasma proteins, which also increases the serum half-life. Another drawback of PS-DNA ODNs is that, compared to unaltered ODNs, they have a little lower affinity for complementary RNA molecules where the heteroduplex's melting temperature is lowered by roughly 0.5^{o}C per nucleotide. This flaw is somewhat made up for by the increased specificity of hybridization observed for PS-ODNs in comparison to unaltered DNA ONs [49].

Another example of a modified phosphate backbone is N3'-P5' phosphoroamidates (NPs), in which a 3'-amino group replaces the 3'-hydroxyl group of the 2'-deoxyribose ring. NPs show nuclease resistance in addition to strong affinity for a complementary RNA strand [54]. Their effectiveness as ASOs has already been shown *in vivo*, where the expression of the c-myc gene in mice was selectively downregulated using a phosphoroamidate ASO. [55]. Consequently, the peripheral blood leukemic load was decreased in mice with severe combined immunodeficiency when myeloid leukaemia cells were implanted. When compared to animals treated with mismatch ASOs, those treated with the ASO agent exhibited a considerably longer duration of survival. Furthermore, NPs were discovered to be more effective than PS-DNA ODNs in the treatment of leukaemia. By sterically preventing translation initiation, phosphoroamidate-mediated antisense effects were shown to be sequence specific in both cell culture and in vivo settings when the target sequence was located immediately upstream of the firefly luciferase initiation codon [56]. NP ASOs may be useful in circumstances when preserving RNA integrity is necessary, such as regulating splicing, because they prevent RNase H from cleaving the target RNA.

1.3.2.2. Second-generation Antisense Oligonucleotides (ASOs)

By including nucleotides with alkyl modifications at the 2' position of the ribose sugar ring, second-generation ASOs (Figure 1.5) partially address the problems associated with PS-DNA ODNs (first-generation ASOs). The two most significant members of this family are 2'-O-methyl (OMe) and 2'-O-methoxy-ethyl (MOE) RNA. These modifications are used to create ASOs which are less harmful than PS-DNA ODNs and have a marginally stronger affinity for their complementary RNAs [57-58]. However, these positive traits are offset by the inability of 2'-O-alkyl RNAs to cause the target RNA to be cleaved by RNase H. According to mechanistic investigations, the right width of the minor groove of the ASO/RNA duplex, flexibility of the ASOs, and accessibility of the RNA's 2'OH group are all necessary for effective RNase H cleavage [59].

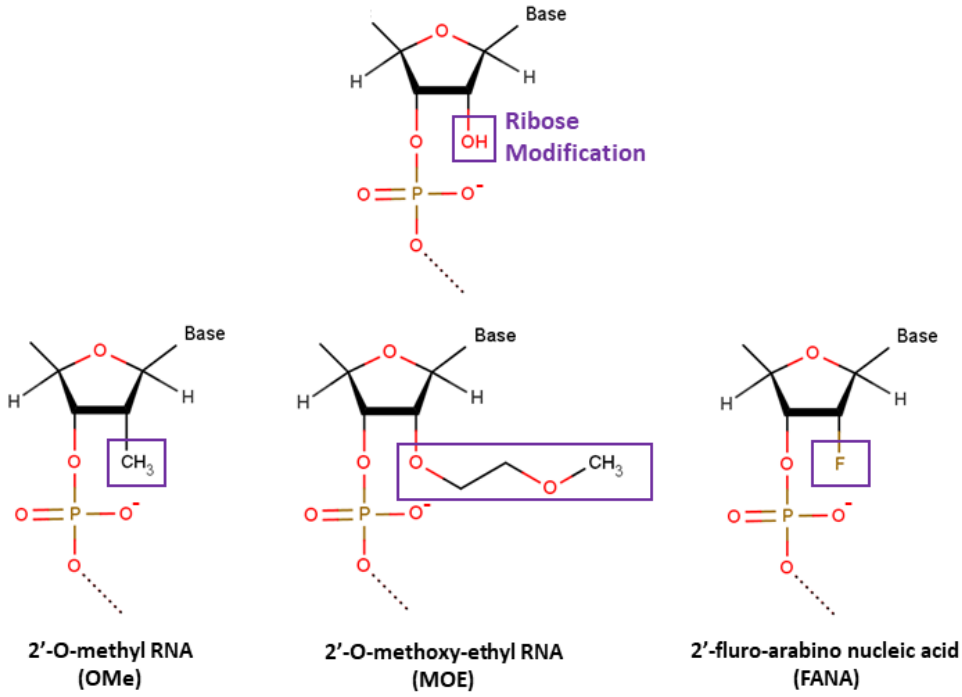

Figure 1.5: Noteworthy Second-generation nucleic acid analogues. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

The antisense impact of 2'-O-alkyl RNA ASOs can only result from a steric obstruction of translation since they do not attract RNase H. The efficacy of this approach was initially demonstrated in 1997, when it was discovered that an RNase H-independent 2'-O-MOE modified ASO that was directed at the 5'-cap region could effectively block the production of the Intercellular Adhesion Molecule 1 (ICAM-1) [60]. This result was most likely brought on by a specific interference with the construction of the 80S translation initiation complex.

Altering splicing is another strategy, wherein ASOs prevent RNase H activation. By inhibiting a splice site, ASOs can increase the expression of an alternatively spliced protein version in contrast to their usual role of decreasing protein expression. In the initial stage of ASO therapeutics, the β-thalassemia genetic blood condition was treated using this method [61]. An error in splicing results from a mutation in the β-globin gene's intron-2 in one variant of this condition, and, consequently, a lack of β-globin. A 2'-O-methyl PS oligoribonucleotide that prevents RNase H cleavage was intended for the incorrect splice place which brought it back to normal β-globin mRNA to correct protein production in mammalian cells.

However, to boost antisense efficacy, target RNA cleavage by RNase H is preferred for majority of antisense therapeutics. ASOs derived from 2'-fluoro-arabino nucleic acid (FANA) were the first to be consistently sugar-modified and to be known to cause a bound RNA molecule to be cleaved by RNase H [62]. It was believed that the fluoro substituent protruded into the helix's main groove, where RNase H shouldn't be affected. Nevertheless, only chimeric ASOs with ODNs in the middle were able to fully activate RNase H, and even then, the DNA stretch required for high enzyme activity was less than that of 2'-O-methyl gapmers [63]. Compared to the equivalent PS-DNA ONs, the chimeric FANA-DNA ONs exhibited a 30-fold lower IC50, indicating their great potency in cell culture.

“Chimeric ASOs” are synthetic molecules designed for therapeutic purposes that combine different nucleotide analogues or modifications in their structure. The term "chimeric" in this context refers to the hybrid composition of oligonucleotides designed to serve several purposes, including enhancing the stability, specificity, and efficacy of the ASOs [64-65]. One common type of chimeric ASO is the “Gapmer ASOs”, which typically consists of a central "gap" of DNA nucleotides flanked by modified RNA or DNA nucleotides (Figure 1.6). The gap is composed of a central stretch of six to eight DNA or PS-DNA monomers, strategically designed to recruit the enzyme RNase H, leading to cleavage of the target RNA. This cleavage prevents the normal translation of the mRNA into a functional protein, providing a targeted approach to modulate gene expression. The flanking regions contains two or more modified nucleotides such as 2'-O-methyl RNA at both ends that cannot recruit RNase H and contribute to the ASO's stability and binding affinity. End blocks prevent nucleases from breaking down the ASOs. By utilizing sequence complementarity to hybridize with transcripts, gapmers

attract the Ribonuclease H and trigger the destruction of target RNA. Gapmers only cause RNase H cleavage of a single target sequence and they can be used to remove extraneous cleavage. Research has demonstrated that human and Escherichia coli RNase H can be activated by a continuous length of four or five deoxy residues between flanking 2'-O-methyl nucleotides [66-68]. The "irrelevant cleavage," another issue related to ASOs, has also been addressed with the introduction of gapmers.

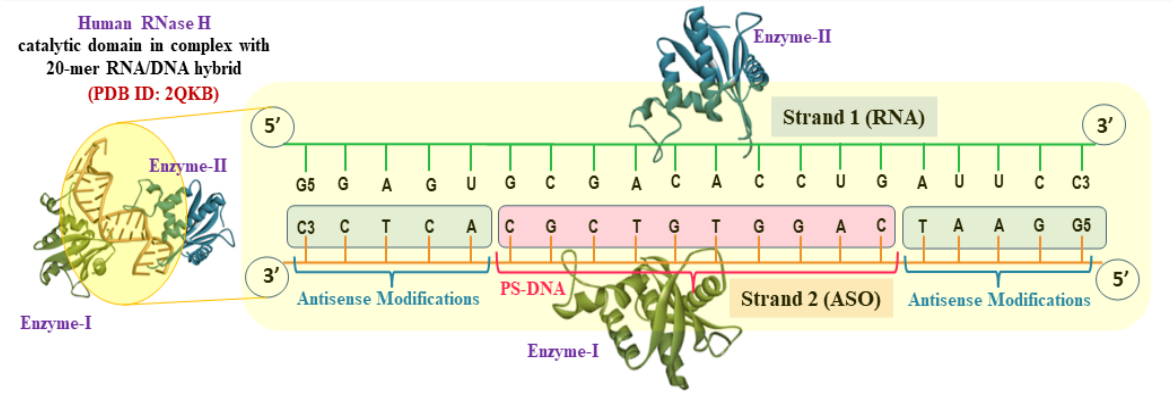

Figure 1.6: Schematic representation of chimeric gapmer concept containing a 20-mer ASO/RNA hybrid duplex bound by a dimeric RNase H.

Gapmers have effectively enrolled in clinical trials for a range of hereditary disorders, many of which have already received FDA approval for treating disorders as familial Hypercholesterolemia and Transthyretin Amyloidosis-associated Polyneuropathy [69]. The FDA approval of drugs like Mipomersen (Kynamro), Eteplirsen (Exondys 51), Volanesorsen (Waylivra), Nusinersen (Spinraza) highlights the clinical success of such chimeric ASOs. In 2013, the FDA approved Mipomersen a 2′-O-methoxyethyl (MOE) gapmer for the treatment of homozygous familial hypercholesterolemia (HoFH) [70-71]. Eteplirsen is an FDA-approved chimeric ASO used to treat Duchenne Muscular Dystrophy. It induces exon skipping during mRNA splicing, allowing for the production of a functional dystrophin protein [72-73]. Another 20-mer MOE gapmer, Volanesorsen, has demonstrated efficacy in reducing triglyceride (TG) levels in a number of lipid disorders [74-75]. This chimeric ASO targets the mRNA of apolipoprotein C-III and is being investigated for the treatment of familial chylomicronaemia syndrome and hypertriglyceridemia. In May 2019, the European Union granted conditional approval for the treatment of familial chylomicronaemia syndrome (FCS), based on the positive outcomes of phase II/III clinical trials. Nusinersen used to treat spinal muscular atrophy (SMA) is an 18-mer chimeric ASO gapmer consisting of 2'-O-methoxyethyl-modified (MOE) nucleotides with phosphorothioate backbone [76-77].

1.3.2.3. Generation 2.5 Antisense Oligonucleotides (ASOs)

Advances in chemistry and molecular biology contributed to the development of more stable and efficient oligonucleotides by utilising the concept of conformational restriction to enhance binding affinity and biostability. Moreover, nucleotides with an entirely different chemical component in place of the furanose ring as well as DNA and RNA analogues with altered phosphate linkages or riboses have been created. Like the "first generation" of phosphorothioate DNA and the "second generation" of 2'-O-alkyl RNAs before them, these new nucleotides are then included in the category of "generation 2.5" antisense agents. A few exciting examples of the large corpus of innovative modified nucleotides with better features include the Peptide Nucleic Acid (PNA), Phosphoramidate Morpholino (PMO), Cyclohexene Nucleic Acids (CeNA), Tricyclo-DNA (tcDNA), Locked Nucleic Acid (LNA) and the Bridged Nucleic Acids (BNAs).

- **Peptide Nucleic Acids (PNAs):** Peptide Nucleic Acids (PNAs) (Figure 1.7) initially described by Nielsen and colleagues in 1991 [78] include polyamide connections in place of the normal deoxyribose phosphate backbone, and, are the most researched DNA analogues apart from PS-DNA and 2'-O-alkyl RNA [79-80]. PNAs are highly biologically stable and have advantageous hybridization properties, however they do not cause RNase H to cleave target RNA. *In vivo* experiments suggests that PNAs are not harmful because PNAs are uncharged molecules with a low affinity for proteins that ordinarily bind nucleic acids. Perhaps the most promising application of PNAs is not as antisense agents but rather as strand invaders of chromosomal duplex DNA to alter gene expression. Furthermore, solubility and cellular uptake are significant obstacles that must be resolved before PNAs may be used as antisense agents because they are molecules that are electrostatically neutral. Combining PNAs with lipids, negatively charged oligomers, or specific peptides that cells can internalize effectively may result in improved intracellular delivery. Given PNA's special qualities such as its resistance to enzymatic digestion, greater biostability, and strong hybridization attraction toward DNA and RNA, PNA-based technology has garnered a lot of interest as a potentially effective method for gene modification, however, inadequate intracellular uptake presents a significant barrier. PNAs have been shown to function mainly as antisense and antigene agents, which impede the transcription and translation of target genes. However, they can also be utilized for more therapeutic purposes, such as modifying splicing patterns and editing genes to alter specific regions of the genome.

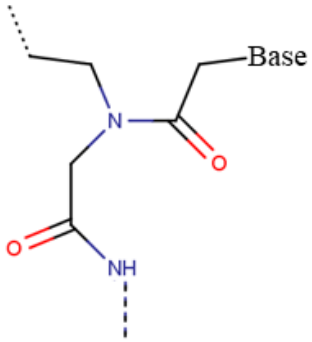

Figure 1.7: Peptide Nucleic Acid (PNA). The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

In one of the early stages of *in vivo* investigations, the PNAs were utilized in a transgenic mouse model to correct faulty splicing [81]. PNAs were targeted at a human β-globin gene mutant intron that disrupted the gene responsible for encoding increased green fluorescence protein (GFP). The expression of functional GFP was observed in the presence of systemically administered PNAs. A 2'-O-methoxy-ethyl was the least active of the ASOs examined, with the exception of the small intestine, where PNAs linked to four lysines at the C-terminus. The four-lysine tail is necessary for PNAs to have antisense action *in vivo*, as evidenced by the fact that PMOs were much less effective and PNAs with just one lysine were completely ineffective.

Recent studies include application of PNAs as anti-infective agent, therapeutic application as anti-miR agent, as gene editing agent, as splicing modulator in clinically relevant approaches, as valuable tools for target-directed cancer therapy based on *in vivo* experimental systems and clinical studies as manipulation of both translation and transcription of *bcl-2* gene *in vitro,* targeting Chronic Myeloid Leukaemia (CML), Acute Promyelocytic Leukaemia (APL), blocking of BL cells expansion and downregulation of *c-myc* expression [82]. Because of its neutral composition, PNAs are poorly soluble in water and tends to form globular forms. The low cellular absorption of this hydrophilic oligomer is another important obstacle. PNAs have no biological activity if they are unable to engage with the target sequence through the cell membrane. Hence, in reality, a lot of thought still needs to be given to issues like immunogenicity, biodistribution, and other drug development-related issues when it comes to *in vivo* delivery.

- **Phosphoroamidate Morpholino Oligomer (PMO):** PMOs (Figure 1.8) are non-ionic DNA analogues in which phosphoroamidate intersubunit connections are utilized in place of phosphodiester bonds and a morpholino moiety is employed in place of the ribose [83-84]. PMOs' uncharged backbone makes it unlikely that they may inadvertently interact with proteins that bind nucleic acids. Their target affinity is similar to that of isosequential DNA ONs, but it is lower than the strength of RNA binding obtained with many of the other analogues. PMOs can be used to decrease gene expression by blocking ribosomes from binding to the 5' untranslated region or the first 25 nucleotides downstream of the start codon. They do not activate RNase H.

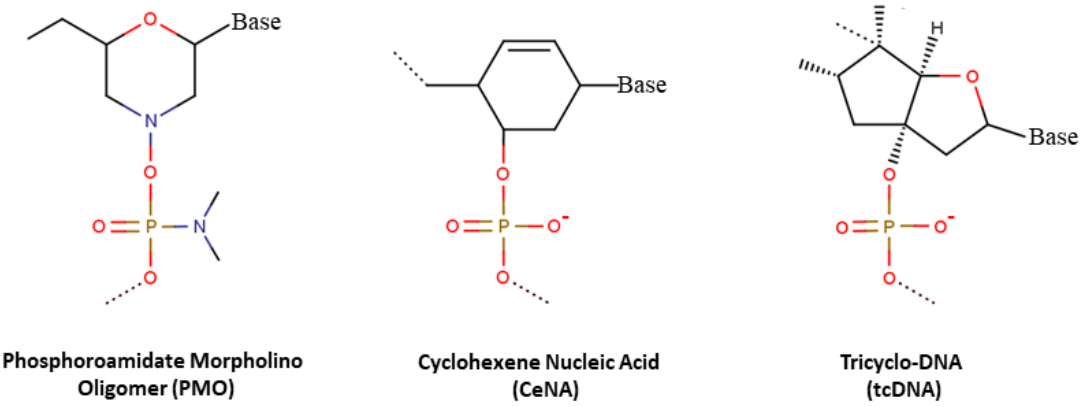

Figure 1.8: Noteworthy generation 2.5 nucleic acid analogues. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

Most published work on PMO compounds has been carried out using zebrafish embryos. PMOs against GFP in a universal GFP transgenic were able to effectively knock down a gene in every cell of zebrafish embryos [85]. In order to explore the regulation of ccnd1, ccnd1-specific 20-mer PMO ASOs were microinjected to inhibit the translation of ccnd1 mRNA, a G1/S checkpoint control element. The cyclin D1 protein was subsequently downregulated, causing microphthalmia and microcephaly but not death [86-87]. A possible medicinal use for PMOs that fixed mutant β-globin precursor mRNA's improper splicing was also documented [88]. mRNA synthesis was restored post correct splicing at the aberrant splicing sites with enhanced haemoglobin when the ASOs were administered to erythroid progenitors from peripheral blood of thalassaemic patients. However, these tests necessitated high ON concentrations and mechanical disruption of the cell membrane due to the restricted cellular uptake of PMOs. Additionally, the cause of the general adverse effects that have been reported in multiple studies is another pertinent subject that has to be addressed.

- **Cyclohexene Nucleic Acid (CeNA):** The foundation for cyclohexene nucleic acids (CeNAs) (Figure 1.8), which are distinguished by a high degree of conformational rigidity of the oligomers, is the substitution of a six-membered ring for the five-membered furanose ring. They shield ONs from nucleolytic destruction by forming stable duplexes with complementary DNA or RNA [89]. It was documented that CeNA/RNA hybrids can activate RNase H, albeit at a 600-fold lower k_{cat} than a DNA/RNA duplex [90]. However, there is still more work to be done in designing ASOs with CeNA to produce highly effective ASO agents.
- **Tricyclo-DNA (tcDNA):** Leumann and coworkers were the ones who initially synthesized tricyclo-DNA (tcDNA) (Figure 1.8), an additional nucleotide with improved binding to complementary RNA sequences [91-92]. Like the other DNA and RNA analogues, tcDNA does not cause the target mRNA to be cleaved by RNase H. However, a mutant β-globin mRNA was successfully corrected by using it with a 100-fold increase in efficiency compared to an isosequential 2'-O-methyl phosphorothioate RNA [93]. The therapeutic potential of ASO made of tricyclo-DNA (tcDNA) were explored to silence huntingtin gene (HTT) which encodes for the toxic polyglutamine protein [94]. Utilizing a gapmer ASO with core DNA nucleotides surrounded by tcDNA alterations on the 5′ and 3′ ends, they were able to attract RNAse H and cause the mRNA to degrade. HTT mRNA and protein levels were found to sharply decline in patient-derived fibroblast cell lines following tcDNA-ASO transfection. This shows that gapmer tcDNA ASO has the therapeutic potential to downregulate huntingtin both *in vivo* and *in vitro*.

Additionally, it has been found that ASOs constructed of tcDNA hold great promise for the treatment of Duchenne Muscular Dystrophy (DMD), which is usually brought on by nonsense mutations or frameshifting deletions in the dystrophin gene. The effectiveness and toxicological profile of tcDNA were also studied in models of mdx mice [95]. The study found that systemic delivery of a 13-mer tcDNA restores dystrophin in the skeletal muscles and, to a lesser extent, in the brain, improving muscle function and correcting behavioural traits associated with the cognitive deficit. More significantly, the effects of tcDNA treatment were typically restricted to small glomerular alterations and a small number of cell necroses in the proximal tubules, with very little change in the levels of kidney toxicity biomarkers in the urine and serum. These findings validate tcDNA's therapeutic promise for the treatment of DMD patients and show an encouraging safety profile, albeit typical of phosphorothioate ASOs.

1.3.2.4. Bridged Nucleic Acids (BNAs)

Bridged Nucleic Acid (BNAs) are modified RNA nucleotides which have a bridge artificially added at the 2' and 4' positions of the ribose to produce restricted RNA molecules. BNA monomers have a fixed *'C3'-endo'* sugar puckering and can have up to five-, six-, or even seven-membered bridging structures. Depending on the kind of monomer, the bridge's composition can change. The design's objective is to identify derivatives with strong binding affinities complementary to DNA and RNA strands.

- **Locked Nucleic Acids (LNA):** Introduced in the late 1990s, the first BNA popularly known as the Locked Nucleic Acid (LNA) (Figure 1.9) is considered as the earliest generation of BNAs making it one of the most promising chemically modified nucleotide possibilities discovered in recent years [96-97] LNAs are bicyclic nucleotide analogues in which a methylene group is added to link the 2'-oxygen and the 4'-carbon (2'-O,4'-methylene-β-d-ribofuranosyl nucleotides) in the furanose sugar ring [98-100]. They are distinguished by a locked *N-type* conformation that promotes the creation of stable duplexes with DNA or RNA and decreased ribose residue flexibility [101].

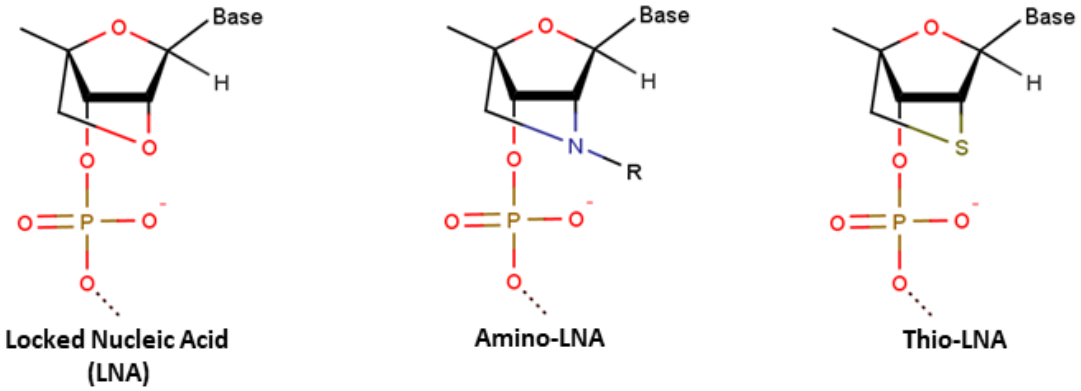

Figure 1.9: Locked nucleic acid (LNA) analogues. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

In the first *in vivo* investigation for an LNA, the rat delta opioid receptor was effectively knocked down with no observable adverse effects in the rat brain [102]. Full LNA ASOs were then effectively employed *in vivo* to prevent the translation of RNA polymerase II's big subunit [103]. In a xenograft model, these ASOs suppressed tumor growth at an effective concentration five times lower than that of the comparable phosphorothioate DNA previously discovered. Back then, the LNA ASOs seemed safe at the recommended dosage. Consequently, a number of desirable characteristics, including

stability against nucleolytic degradation, high target affinity, strong biological activity, and an apparent lack of acute toxicity, seem to be offered by complete LNA ASOs. Later on, it was observed that fully modified LNA ASOs prevents RNase H from cleaving the target RNA [104]. Human telomerase is a ribonucleoprotein with an RNA component that hybridizes to the telomere that is expressed in tumor cells but not in normal tissues. As such human telomerase was then sought to be a potential target for ASOs containing LNA. Since RNA degradation is not required to obstruct the catalytic region of the enzyme, LNA ASOs that are unable to recruit RNase H should be able to access telomerase and were sought to be appropriate telomerase activity inhibitors. According to comparative research, LNAs were far more likely than PNAs in inhibiting human telomerase [105].

Since the goal is to degrade the mRNA, chimeric LNA-DNA gapmer should be utilized, which has DNA monomers in the centre to activate RNase H [106]. Compared to PS-DNAs or 2'-O-Me modified gapmers, chimeric LNA-DNA ASOs have a far higher effectiveness in reducing gene expression in cell culture due to their improved affinity towards the target RNA, which also speeds up RNase H cleavage. They have a remarkably high target affinity and improved stability against nucleolytic degradation. Targeted to different locations within the luciferase mRNA, LNA and LNA-DNA chimeras effectively suppressed gene expression [107]. It was reported that chimeric 2'-O-methyl-LNA ASOs as steric blocks prevented intracellular HIV-1 Tat-dependent trans activation and decreased gene expression, even if they do not activate RNase H [108]. The increased antisense efficacy of LNA was proposed to be explained by enhanced cellular uptake of ASOs comprising 2'-O-methyl RNA and LNA, as opposed to an all 2'-O-methyl RNA oligomer. This was an addition to target affinity. Therefore, LNA ASOs are typically produced as chimeras, which combine LNA with RNA, DNA or other nucleotide analogues [109].

When compared to the isosequential strands of unmodified compounds, several structural and thermal stability investigations on complexes made of complementary DNA or RNA oligonucleotides and LNA oligomers revealed higher melting temperatures and specificity [110-111]. Majority of chimeras containing LNAs fall into one of the two categories: gapmers or mixmers. Gapmers are made up of oligomers with the LNA residues at the ends of the strands. The LNA and the other residues scattered throughout the sequence in various combinations are mixmers.

LNA-containing ASOs have been employed in varied dimensions as gene repair [112], antisense medications [113-117], hybridization, amplification, mutagenesis, sequencing, SNP genotyping, and diagnostics [118-121]. Higher binding capacities can be detrimental as well, since they can produce duplex configurations that the enzymes needed to degrade the target molecule cannot recognize as substrates, despite the fact that they are helpful in many antisense applications. Additionally, due to non-specific off-target binding, the high affinity of LNA ASOs can also have harmful effects. Some research articles demonstrated that LNA-containing ASOs are safe for humans [122-123] and harmless for primates [124] and that these substances did not exhibit genotoxicity [125]. However, these findings are by no means conclusive, as other research demonstrated hepatoxicity of LNA-containing ASOs [126-128]. While the toxicity assessments of LNA-containing ASOs are improving, development as human illness therapeutics will depend on case-by-case analysis.

The poor absorption of LNA ASOs by bacterial cells poses a challenge to their development as medicines, especially in the context of silencing prokaryotic genes. There are few instances of the use of other uptake-promoting techniques to suppress gene expression, such as conjugation to Cell Penetrating Peptides (CPP) [129]. LNA-containing ASOs were also introduced by nanoparticles into target cells [130]. Cancer cells exposed to an 8-nucleotide LNA oligomer that is complementary to the oncogenic *miR21* present in micelles induced apoptosis. Additionally, in an animal model, these studies demonstrated suppression of tumor growth.

Many such medications based on LNAs including ISTH0036, MRG-106, and Miravirsen, are currently undergoing clinical trials [131-132]. In clinical trials, PS-LNA ASOs caused severe thrombocytopenia, nephrotoxicity, hepatotoxicity, and injection site reactions (ISRs) although no information regarding the mechanism causing this thrombocytopenia has been made public. All tested PS-LNA ASOs induce moderate self-limited platelet decreases at doses >200 mg/week. Compared to other 2′ modified PS medications, PS-LNA ASOs exhibited more toxicity in *in vitro* tests, mice, and NHPs. Since numerous PS-LNA ASOs were demonstrated to be harmful in the clinic, as a result, there aren't any active clinical trials using ASOs from this class of LNAs. To sum up, compounds containing LNA have a lot of potential as therapeutic agents. However, more study is necessary to increase cell penetration while maintaining their biological activity and decreased toxicity.

- **Constrained Methoxyethyl (cMOE) and Constrained ethyl (cEt):** Some ASOs have been found to be more potent in animals when 2′-O-MOE residues are replaced with LNAs; nevertheless, this comes with a markedly increased risk of hepatotoxicity. It was then proposed that the toxicity of LNA might be reduced without sacrificing efficacy by substituting new nucleoside monomers that mix the structural components of MOE and LNA [133-135]. A series of nucleoside modifications were produced by combining the structural elements of 2′-O-MOE and LNA nucleosides of which cMOE (2′,4′-constrained MOE) and cEt (2′,4′-constrained ethyl) modifications (Figure 1.10) were found to have better potencies than MOE ASOs and a better therapeutic index than LNA ASOs. In R-cMOE (R-constrained MOE) and S-cMOE (S-constrained MOE) the ethyl chain of the 2′-O-MOE moiety is constrained back to the 4′ position of the furanose ring. In R-cEt (R-constrained Ethyl) and S-cEt (S-constrained ethyl) the methoxymethyl group in the cMOE nucleosides is replaced with a methyl substituent.

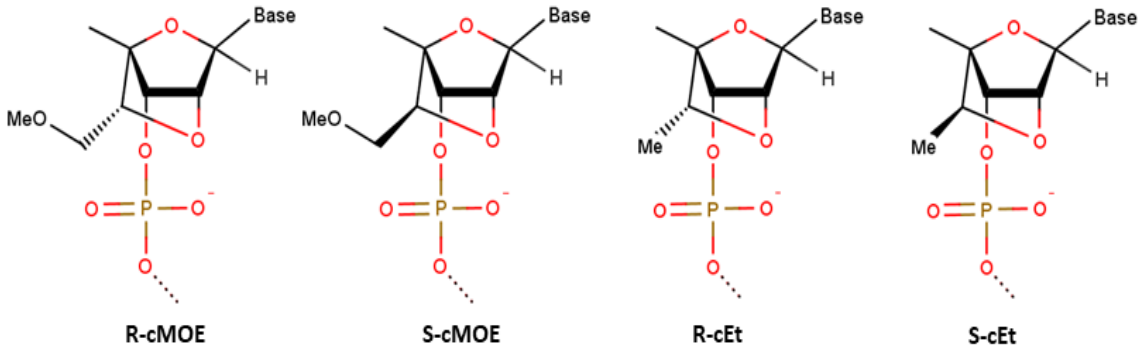

Figure 1.10: Constrained Methoxyethyl (cMOE) and Constrained ethyl (cEt) nucleic acid analogues. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

Animal tests with the cMOE and cEt modifications revealed that all of them had much improved toxicity profiles over LNA and were much more potent than second generation MOE ASOs [136]. cEt modified ASOs were studied in mice and monkey to support oncology clinical trials targeting signal transducer and activator of transcription 3 (STAT3) and their toxicity profile was very similar to that of 2′-MOE-ASOs. Although less explored than the LNAs, these alterations were studied elaborately to observe anti-tumor effects in syngeneic tumor models [137], across human cancer cell lines [138], hybridization-mediated off-target effects of splice-switching [139], implications of hepatocellular carcinoma growth [140], showcasing the pharmacokinetic behaviours of 2′ PS MOE, PS 2′-cEt, and PS 2′ LNA ASOs to be quite similar.

- **2′,4′-BNA^{NC}:** The popularity and benefits of LNA-containing ASOs for a variety of uses prompted researchers to look for related substitutions with better qualities. This led to a number of derivatives to be introduced, such as 2′-O,4′-C-ethylene-bridged nucleic acid (ENA) [141], 2′-O,4′-C-methylenoxymethylene-bridged nucleic acid [142-144] and 2′,4′-BNA^{NC} with various substitutions at the N atom (Figure 1.11) (of which a methyl group is the most commonly used to date) [145]. The number of members in the ring of the bridge might vary between the many BNA compounds; the ring of BNA^{NC}, which has six members, is the most commonly employed to date [146-147].

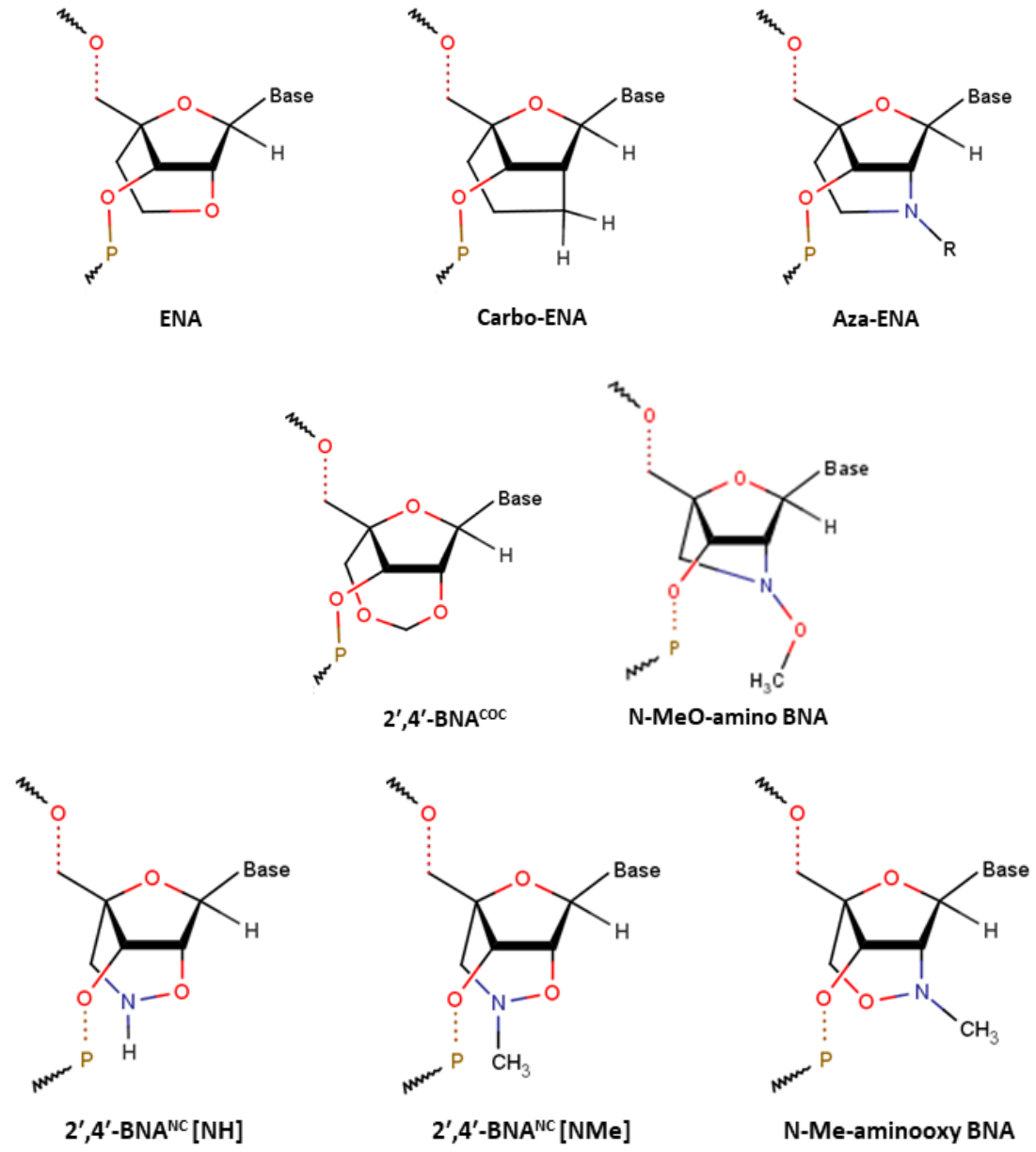

Figure 1.11: Noteworthy 2′,4′-BNA nucleic acid analogues. The Base denotes one of the bases Adenine, Guanine, Cytosine or Thymine.

Following years of research and development, it has been discovered that the 2′,4′-BNA^{NC} analogues: 2′,4′-BNA^{NC}[NH], 2′,4′-BNA^{NC}[NMe], N-Me-aminooxy BNA, and N-MeO-amino BNA alterations show great promise for use in antisense technology. *In vitro* and *in vivo* studies revealed that optimized BNA ASOs outperformed the MOE ASOs in terms of thermal stability, enhanced *in vitro* activity, and >five-fold higher *in vivo* activity [148-149]. Toxicity markers AST, ALT, liver, kidney, and body weights, were also shown to be normal for N-MeO-amino BNA, N-Me-aminooxy BNA, and 2′,4′-BNA^{NC} ASOs. When duplex stabilities were compared with corresponding single-stranded RNA (ssRNA), 12-mer ASOs with varying numbers of BNA^{NC} or LNA residues demonstrated a similar rise in melting temperature (Tm) per changed residue (4.7 to 7.0 ◦C) [150]. These findings suggest that the antisense oligomers had a greater affinity for RNA-specific binding due to the presence of BNA^{NC} residues. In addition, BNA^{NC}-containing oligonucleotides displayed more stable triplexes and better mismatch discrimination properties than LNA-containing oligomers [151]. According to these findings, chimeric gapmers modified with 2′,4′-BNA^{NC}[NH], 2′,4′-BNA^{NC}[NMe], N-Me-aminooxy BNA, and N-MeO-amino BNA may be suitable as therapeutic candidates for use in antisense drug discovery platforms.

Thus, in comparison to isosequential compounds having LNA residues, oligonucleotides containing 2′,4′-BNA^{NC} residues exhibited greater resistance to nucleases, are less toxic, have good thermal stability and water solubility, and cause RNase H to degrade target RNA [152]. Stability and specificity were dramatically improved by the substitution of RNA, DNA residues with BNA^{NC} analogues. They were also discovered to have good single-mismatch discriminating capacity, stable triplex-forming characteristics, and greater sequence selective binding affinity for complementary RNAs.

As is the case of numerous BNA molecules, BNA^{NC}-containing oligonucleotides have the potential to be utilized in diverse applications and some are currently or soon will be accessible for testing [153]. BNA^{NC} modifications has been dealt with by conjugating the compounds to permeabilizing peptides, using transfecting agents, packaging them into liposomes, and by combining them with nanoparticles. Challenges in the development include optimizing the specificity of the BNA^{NC}'s, ensuring effective delivery to target cells, and addressing potential off-target effects. A few cases of such applications are discussed in the subsequent sections.

1.4. Computational Platforms for Antisense Oligonucleotides (ASOs)

For successful implementation of ASO therapeutics one would need to identify target RNA sequences, design ASOs to complement them, and predict the binding affinity and specificity of the ASOs. The computational platform for ASOs involve bioinformatics and molecular modelling tools. Computational tools like RNA folding algorithms can help predict the secondary structure of RNA, which is crucial for ASO design. Molecular Dynamics (MD) simulations might come into play to predict how well the ASOs bind to their target RNAs. Significant advancements in the field of computation have been made in recent times with the creation of advanced softwares, force-fields, parameterization protocols including analysis plots, thermodynamic indices, binding energy histograms etc. And, of course, there's always room for machine learning to analyse data and improve the efficiency of ASO design.

ASO sequences can be chosen by computer programs according to the target gene or mRNA sequence. This might result in the creation of more selective ASO sequences. In the early 1990s, *Stul et al.* created three thermodynamic indicators (Sscore, Dscore, Cscore) to assess probable structure complexes involved in ASO activity, which would help in the selection of the best ASO targets [154]. The genomic frequency of di-, tri-, and hexamers found in a particular target sequence was calculated using the computer program Oligomer [155]. The distribution of hexamers inside the genome was discovered to be non-random suggesting that non-specific binding may be reduced by choosing antisense oligonucleotide sequences against fewer common hexamers.

Since it is unclear what sequence characteristics make an ASO effective, it is necessary to test a large number of ASOs in order to identify effective inhibitors. Earlier computer models solely relied on RNA structure prediction or motif searches, disregarding data from other ASO design factors. Nearly 20% or less of the ASOs are usually effective inhibitors *in vivo* if the target site in the mRNA is chosen at random. *Chalk et al.* developed a neural network-based computational model that can predict ASO with a wide variety of input parameters [156]. They built a neural network model using generated parameters based on features of ASO sequences. With a 92% success rate, this model forecasted effective ASOs (>50% suppression of gene expression) and predicted 12 effective ASOs per 1000 base pairs on average making it a strict but useful technique for ASO prediction. In another work by *Anusha et al.* described an Artificial

Neural Network (ANN) based computational technique that can effectively recognize and forecast oligonucleotides [157]. 11 key characteristics related to oligo/mRNA duplex were found. The input sequences are precisely predicted by a classifier into one of two classes: oligo or non-oligo. After validation, their tool showed a very significant 92.48% accuracy, 91.7% sensitivity, and 92.09% specificity.

Far et al. examined in detail the connection between the hit rate of the biological efficiency of ASOs in cell culture and the computational parameters of the local target search for the theoretical design of ASOs [158]. Using an established algorithm to identify structurally favourable local target locations along a given target RNA, the foundation for the computational design of ASOs was examined where a set of lowest-energy RNA secondary structures is predicted from a sequence segment of a given length. The systematic structural target analysis's technical parameters have been selected at random, examined their significance in the effective creation of ASOs and proposed an enhanced computer-based approach to identify advantageous local target sequences, leading to a better design of active ASOs.

Many computational and experimental strategies have been put forth since designing antisense ODNs requires locating efficient target sites within RNA. *Smith et al.* predicted secondary structure of mRNA and RNase H mapping and use of combinatorial arrays by computational methods [159]. Valid and invalid ODNs reported in literature were filtered, gathered, and stored in AOBase to facilitate better sharing of the tested and published ODNs [160]. PFRED is a newer informatics platform for designing, analysing, and visualizing siRNA and ASOs [161]. Scientists can create a library of ASOs that target a particular gene of interest using the software's user-friendly interface. Additionally, the tool makes it easier to include different design parameters that have been demonstrated to be crucial for potency and stability. Skilled developers can also add unique or proprietary descriptors.

MD simulations were initially used to simulate proteins and it is only a few years later that nucleic acids were included in the history of MD simulations [162-165]. It is now evident that realistic and accurate simulations require the inclusion of long-range electrostatic interactions as well as the aqueous and ionic environment. One can obtain pertinent information about the impact of the nucleotide sequence and different solvent conditions on the structure and dynamics of molecules as large as tRNA through stable

and seemingly realistic simulations of nucleic acids over several nanoseconds. Results are already contributing to the understanding of how nucleic acids react to their surroundings and to their base sequence. They are also revealing the factors that underlie recognition processes. The ability to closely monitor processes like conformational transitions and closely follow the behaviour of solvent molecules and their interactions with the solute are two important benefits of the extremely detailed information obtained in simulations. Outstanding issues include simulating considerably larger molecular systems and treating chemical events, as well as simulating very slow and intricate processes like the folding of RNA domains or the synthesis of nucleic acid-protein complexes. However, improving dependence of the outcomes on force-field protocols, thereby containing the scope of future developments that will be required to enhance our understanding of recognition phenomena and nucleic acid folding is a necessity.

While evaluating the computational techniques, *Sponer et al.* reported quantum chemical calculations of molecular interaction in nucleic acids and RNA structural bioinformatics targeting combined application of contemporary *ab-initio* methods and bioinformatics analysis of experimental structures [166]. Their ultimate goal was to acquire important physicochemical understandings of the nature of the underlying molecular interactions and how they influence the dynamics, structure, function, and evolution of RNA. The physical chemistry constraints of the limited time scale and force field (FF) approximation in molecular mechanics (MM) were also clarified by *Sponer et al.* [167]. They compared normal and enhanced sample simulations, the relationship and differences between simulations and experiments, the role of beginning structures, nucleic acid FF versions, and connected MM computations with modern quantum chemistry. From the results it was established that despite its drawbacks, MD is an effective method for researching the structural dynamics of nucleic acids with a rapidly expanding potential that significantly enhances and facilitates the interpretation of experimental data.

As a promising class of antibacterial drugs, *Panecka et al.* conducted MD simulations of 2′-O-Me and PNA modified ASOs complementary to bacterial ribosomal A-site RNA [168]. Their results consist suggesting that substituting thymine for uracil results in a less flexible PNA counterpart and 2′-O-methylation inhibits the dynamics of the RNA backbone, preventing interconversion of stacked and unstacked conformations. Localization of water molecules was higher in 2′-O-Me than in PNA with highest Na^+

density for 2′-O-methyl and Na^+ being essentially non-existent for the neutral PNA systems. *Jasiński et al.* created force field parameters for MD simulations of PNA-involving duplexes at room temperature and high temperature to study the thermal stabilities of short PNA/PNA, PNA/RNA, and RNA/RNA duplexes [169]. The results replicate the thermal stability from melting studies and display a two-state melting transition, with PNA/PNA having the highest activation energy for melting. According to the atomistic details, all PNA/PNA bases melt simultaneously, while RNA/RNA is the least stable and PNA/RNA becomes destabilized as it moves from the termini to the center region of the duplexes. The elastic characteristics of the PNA/DNA and PNA/RNA hybrid duplexes fall between those of dsPNA and dsDNA, dsRNA [170]. While PNA/PNA and PNA/RNA unwind upon stretching like dsRNA, PNA/DNA hybrids overwind similarly to dsDNA. PNAs were then investigated to create a novel class of compounds that might specifically target the 3′-UTR on MYCN mRNA, which functions as a tumor suppressor in neuroblastoma and other malignancies [171]. Three PNA-based oligomers of varying lengths were designed and synthesized, and their interaction with two binding sites on the target MYCN mRNA was examined using MD simulation. The PNA/RNA duplexes maintained intriguing properties in terms of stability, affinity, and cellular uptake even in the face of several mismatches.

Palindromic 8-base pair PNA duplexes were simulated by *He et al.* in explicit water, and the results showed that PNA structures correspond to a P-type helix with helical parameters that are similar to those shown in PNA crystal structures [172]. Again, MD study was conducted on a γ-methylated, palindromic, 8-base pair peptide nucleic acid (γ-PNA) duplex in order to investigate the effect of the γ-backbone modification on the PNA duplex structure [173]. Based on these simulations, it was determined that the γ-PNA duplex follows a general P-form helical structure, which is comparable to that of non-modified PNA but has a minor helical bending towards the major groove and a smaller base pair rise, which is an *A-like* helical feature. When more substituents are added at the PNA's γ-position, the steric limitation that the γ-methyl appears to exert on the backbone orientation is anticipated to become apparent because it is mostly independent of base pairing and stacking.

Wang et al. reported DFT studies on PNA-Pt complexes analysing the nature of high-lying molecular orbitals on PNAs [174]. Based on MD sampling of the PNA structure, *Wolak et al.* performed electronic structure calculations on Cys-PNA SAM,

yielding the electronic density of states and the HOMO-LUMO gap for PNA [175]. The inclusion of a cyclohexyl ring in the PNA backbone causes rigidification of the backbone and a preference for RNA over DNA, as demonstrated by the results of MD simulations reported by *Sharma et al.* [176]. Using DFT, *Bhai et al.* described the function of PNAs and DNA backbones for complexation with various metal ions [177]. According to the computed results, in PNA/DNA duplexes, these metal ions would favourably bond with the DNA over PNA. The contribution of charged backbones as opposed to neutral PNA backbones is primarily responsible for the preference for the interaction of metal ions with DNA nucleotides. By substituting one or two methylene groups on either side of the phosphate backbone for the 2′-deoxyribose in a series of chemically modified analogues, *Murillo et al.* used MD simulations to systematically test these analogues' ability to form stable duplexes with both DNA and RNA [178].

1.4.1. Molecular Modeling Studies on Bridged Nucleic Acids (BNAs)

At the beginning it was proposed that two forms of LNA alterations result in structures that resemble the DNA and RNA are 2′-O,4′-C-methylene-β-D-ribofuranose (β-D-LNA) & 2′-O,4′-C-methylene-α-L-ribofuranose (α-L-LNA) respectively. *Peterson et al.* performed MD simulations on LNAs where they examined a partially modified α-L-LNA/RNA hybrid as well as a fully modified hybrid with the same base makeup [179]. Starting from a typical *A-form* geometry MD simulations were run for 1 ns using AMBER6. The hybrids' backbone angles suggested that the malleable DNA backbone modifies itself to provide the α-L-LNA nucleobases for effective stacking and *Watson-Crick* base pairing. Sugar pucker distribution of the 3′-flanking sugar rings were slightly organized in a more *S-like* shape by the α-L-LNA nucleotides. Whilst LNA can be called a paragon *A-type* mimic, in light of the results obtained from their findings α-L-LNA was assumed to be an ideal *B-type* mimic DNA type.

Suresh et al. also investigated the α- and β-LNA modifications [180]. According to the results, the β-LNA duplex largely departs from both *A*- and *B-form* nucleic acids, while the α-LNA duplex largely resembles the general structure of a typical *A-form* nucleic acid. The helical structure of both LNA duplexes unwinds in contrast to pure DNA and RNA duplexes. Their sugar rings are conformationally comparable to one other, which contradicts earlier ideas that α-LNA substitutions produce structures like DNA. According to free energy estimates, α-LNA or β-LNA significantly increase the

structures stability when compared to pure duplexes. A contributing factor to the increased stability following LNA substitution is demonstrated to be the π-stacking interaction between bases from complementary strands. The striking differences between the duplexes in terms of their structural, dynamic, and energetic properties are suggested to be caused by a combination of two factors: the nature of the -O-CH_2-linkage in the LNAs vs their absence in the pure duplexes, and similar conformations of the sugar rings in DNA and α-LNA vs the other two. Principal component analysis (PCA) and binding free energy approaches were additionally used in a number of geometrical and energetic investigations on α-/β-LNA modifications carried out by *Suresh et al.* [181]. The DNA/RNA duplex undergoes systematic conformational changes upon β-modification, while α-modification modifies the structure rather than maintaining the *A/B-like* conformation. The qualities of partially modified duplexes are midway between those of full strand and unmodified duplexes, while completely modified duplexes have unique properties from partial and unmodified duplexes. The findings indicate that whereas considerable backbone rearrangement is required to incorporate α-LNA modification, which subsequently modifies the energetic and structural features of the duplexes, minimal backbone rearrangement is required for β-LNA modification in order to fit it in duplexes. The simulation results also imply that the site of the modification and the distance between the alterations determine how the characteristics of the DNA/RNA hybrid are altered.

Ivanova et al. subjected MD simulations to a set of LNA-modified DNA 9-mer duplexes, and the resulting structures were examined in terms of base step parameters and sugar residue conformations [182]. A gradual transition from B-DNA to A-DNA helix was identified tracking the impact of an increasing number of LNA nucleotides. The duplex with a one complete LNA strand showed an A-DNA-like structure and any structural disruption brought on by the LNA nucleotide is essentially limited to the base pairs that are directly adjacent to each other inside a duplex. Additionally, in line with the transition to the A-DNA helix, the quantity of *N-type* sugars of nonmodified nucleotides increased with the proportion of LNA nucleotides. Consistent with experimental results, unmodified nucleotides along the 3′-side of LNA nucleotides exhibited a greater propensity to change their sugar conformation from *S*- to *N-type*. The computational protocol was validated by the strong agreement between the relative populations of the sugar conformations and the NMR data.

Pande et al. characterized the hydration of five isosequential LNA/DNA, LNA/LNA, LNA/RNA, RNA/DNA, and RNA/RNA duplexes using MD simulations [183]. The LNA/DNA and LNA/RNA duplexes are structurally identical to RNA/DNA and RNA/RNA duplexes; however, the LNA/LNA duplex has a partially unwinding helix and differs from RNA/RNA duplex in several other respects. RNA/DNA and RNA/RNA duplexes have shorter inter-strand phosphate distances on average than duplexes containing an LNA strand. It was discovered that the intra-strand phosphate lengths in LNA strands are marginally shorter than those in RNA and shorter than those in DNA. LNA is more effective than RNA at adjusting the sugar puckers in partner DNA strands toward *C3'-endo* conformations and the RNA/RNA duplex exhibits greater backbone flexibility than the LNA/LNA duplex. The backbone of DNA is slightly more flexible than that of LNA or RNA, and LNA does not exhibit any conformational changes onto partner strands due to its lesser backbone flexibility. Lastly, although LNA has a well-organized water structure, it is less hydrated than either DNA or RNA. LNA form the most H-bonding bridges, leading to a net higher occupancy of water bridged backbone, whereas DNA, RNA very seldom forms multiple H-bonding bridges via water molecules. A fully modified LNA strand differs from an analogous RNA strand just slightly, yet these little deviations collectively give LNA certain special physical features. These simulations models potentially offered geometrical properties like the amplitudes of sugar ring puckering of LNAs that are not accessible through experimental methods.

A detailed comprehension of the thermodynamic features of duplex formation is essential for the development of stable and effective medications for ASO-based therapies. Using the correlation between the experimental Tm and the estimated duplex binding energy (BE) for ASOs of a particular length, one can potentially estimate the melting temperature (Tm) for nonstandard oligonucleotides. *Shen et al.* devised an automated methodology to determine the thermodynamic parameters of both chemically changed and unmodified oligonucleotide duplexes using BE computation and CHARMm MD simulations [184]. Using the CHARMm component in Discovery Studio (DS), this technique was automated into a standardized MD methodology through Pipeline Pilot (PP) and the computed BE had a strong correlation with the observed Tm. It can be applied in a length-dependent manner to predict the Tm for both unmodified and chemically modified siRNA and ASOs. The method does offer guidance on how to prioritize and choose novel oligonucleotide modifications for additional research before

time-consuming synthesis in siRNA and ASO therapeutics. *Chaubey et al.* studied the relative stabilities and binding interaction between an oligomeric portion of human telomeric DNA [d(TAGGGT)] generating various monomeric quadruplex structures [d(TAGGGT)4] using MD simulations [185-186]. For these investigations, locked nucleic acid (LNA) was used and according to the energy study, a mixed hybrid type quadruplex conformation where two parallel and two antiparallel strands are bound together is more stable than other conformations.

The way that LNA substitutions in a duplex polypyrimidine strand promote triplex formation was investigated by *Xu et al.* [187]. They demonstrated that alternating LNA with one or more non-modified DNA nucleotides pre-organizes the hetero duplex toward a triple-helical-like shape. This in turn encourages the creation of triplexes, whereas subsequent LNAs disrupt the duplex structure to the detriment of triplex formation. The findings are consistent with the theory that a pre-organization in the hetero duplex structure improves triplex forming oligonucleotide binding.

For evaluating if MD simulations can accurately replicate LNA attributes, *Condon et al*. offered a more straightforward benchmark [188] and LNA-specific χ torsional and RESP parameters were developed. They produced AMBER parm99_LNA, new techniques for determining χ torsion ranges and grading the NMR agreement of the simulation are presented. MD predictions using AMBER parm99 and parm99_LNA were compared to a 200 ms NOESY NMR spectra of L(CAAU) and Parm99_LNA and parm99 offer 66% and 35% agreement, respectively, with NMR NOE volumes and 3J-couplings in simulations that begin with an *A-form* structure. In L(CAAU) simulations, parm99_LNA is the only one capable of repairing the structure, with all χ torsions starting in a *syn* conformation. Their results suggests that important interactions can be reasonably approximated by forcefield parametrization techniques for nucleic acid mimics, and that parm99_LNA will increase the dependability of MD research for systems containing LNA.

Martinez et al. utilized MD simulations in conjunction with hybridization analysis to comprehend the structural characteristics of modified ASOs in stabilizing duplex and triplex structures [189]. They looked into the significance of the number and location of LNA substitutions in the ASOs when aiming for an FXN (Frataxin) or c-MYC sequence. It was discovered single strands containing LNA are conformationally

pre-organized for binding to main grooves. While the presence of Twisted Intercalating Nucleic Acid at the 3′-end improves the rate and extent of triplex formation, reduced content of LNA at successive sites at the 3′-end destabilizes the triplex structure. Because ribose nucleotide breaks down quickly, LNA has been proposed as a possible replacement for RNA in the application of RNA-TFO in blood clearance in cellular processes. *Mall et al.* also created various recombinant triplexes by replacing LNA in two distinct DNA sequences and confirmed the stability and ascertained TFO's binding affinity with the natural DNA duplex [190].

The structural and thermodynamic characteristics of whole DNA, RNA, LNA, and 2′-O-methyl RNA duplexes as well as hybrid systems like LNA/RNA, 2′-O-methyl RNA/RNA, LNA-2′-O-methyl RNA/RNA, and RNA-2′-O-methyl RNA/RNA duplexes were examined by *Yildirim et al.* using MD simulations [191]. Modified Amber forcefield tailored for RNA and LNA residues was used for the MD simulations. According to their findings, when LNA and 2′-O-methyl RNA residues are included in hybrid duplexes with RNA, they exhibit two distinct hybridization mechanisms; the former underwinds the duplexes, while the latter overwinds them. Furthermore, the addition of LNA residues to the duplexes gradually reduces base-pair stacking in the LNA and LNA/RNA hybrid systems, but has no effect on the 2′-O-methyl RNA systems. While 2′-O-methyl RNA residues are more flexible and preserve base stacking that is nearly intact in comparison to RNA, LNA residues have a frozen sugar backbone that provides entropic enhancement of stabilities, despite the fact that both types of RNA residues have *C3′-endo* sugar puckering. As a result, 2′-O-methyl RNA modifications boost RNA duplex structural stabilities less than equivalent LNA modifications. Gapmer/RNA oligomer duplexes (LNA-PS-LNA/RNA, RcMOE-PS-RcMOE/RNA, ScMOE-PS-ScMOE/RNA, MOE-PS-MOE/RNA, PS-DNA/RNA, and DNA/RNA) were simulated by *Uppuladinne et al*. [192]. The nucleotides of the corresponding duplexes, MOE and LNA, exhibited the *A-form* helix structure, while certain helical characteristics of the PS-DNA nucleotides suggest that they approximate the *B-form* helix.

Ghobadi et al. investigated the effects of changing the backbone chemistry of ASOs on their hybridization and melting thermodynamics [193]. To facilitate the study, naturally occurring DNA and its chemically modified equivalent PNAs and LNAs were subjected to coarse-grained (CG) model with configurable parameters. These kinds of CG models, together with MD simulations in implicit solvent and with explicit ions,

yielded DNA melting curves that agreed with those obtained with empirical nearest-neighbour models. The impact of backbone flexibility, charge, and nucleobase spacing along the backbone on the melting curves, potential energy, and conformational entropy change upon hybridization, as well as base-pair hydrogen bond residence time, are then clarified using these CG simulations. We discover that duplex thermal stability and melting temperature are reduced when backbone flexibility increases, mostly as a result of higher conformational entropy loss during hybridization. Eliminating charges from the backbone results in a higher energy gain following hybridization, which in turn improves duplex thermal stability by removing electrostatic repulsion. Stacking interactions are critical to duplex stability and increasing nucleobase distance reduces duplex thermal stability.

Likewise employing the AMOEBA polarizable forcefield and the free energy perturbation technique, *Jing et al.* [194] investigated the effects of PS, LNA and PNA on DNA analogues. The preorganization process for LNA and PNA explains the increased duplex stability. In DNA/RNA hybrids, Sp is less stable than Rp because the S and R isomers (Sp and Rp) of PS prefer *C2'-endo* and *C3'-endo* sugar puckering conformations, respectively. Furthermore, the destabilizing effect of Rp is explained by its solvation penalty. Since the locked sugar ring dominates the sugar puckering, PS-LNA and LNA are identical.

Because of their extreme flexibility, single-stranded ONs can take on a variety of conformations in solution, some of which might not be favourable for hybridization. The rates of association (k_a) and dissociation (k_d) govern the binding affinity of therapeutic ONs for their cognate RNA. *Rajasekaran et al.* examined the possibility of modifying the hybridization kinetics of the modified ONs for complementary RNA by limiting rotation around the sugar-phosphate backbone by the use of hydrocarbon bridges to tether two neighbouring backbone phosphonate esters [195]. A ring-closing metathesis reaction was used to assemble the backbone-constrained nucleotide trimers with stereo defined configurations at the contiguous backbone phosphorus atoms. Phosphoramidites synthesized *in situ* were coupled to solid supports to form ONs. After the modified ONs were evaluated, it was found that the 15-membered macrocyclic-constrained analogues had much higher off-rates than the unmodified DNA ONs, which led to decreased duplex stability. Alternatively, LNA ONs with furanose rings that were conformationally preorganized exhibited on-rates that were comparable to DNA ONs, but with very slow

off-rates, leading to a net improvement in duplex stability. Moreover, the results of the MD simulation were largely confirmed by the experimental data, indicating that this approach can be utilized as a predictive tool to develop the next generation of restricted backbone ON analogues with better hybridization capabilities.

The G-rich tract of the LNA-modified DNA aptamer RNV66 and its unmodified counterpart V7t1 can fold into G-quadruplex structures. Both aptamers target the vascular endothelial growth factor (VEGF), which is linked to carcinogenic angiogenesis. The reason behind the polymorphic structure of V7t1 and the distinctive quadruplex fold, increased nuclease resistance, thermal stability, and greater binding affinity for VEGF, of RNV66, its counterpart changed by LNA, is unknown. *Pal et al.* examined the structural and dynamic effects of the insertion of LNAs using MD simulations of RNV66 and V7t1 [196]. Using OL15 for the DNA aptamer and parm99_LNA, along with parmbsc0 and βOL15 for the LNAs, and combining different torsional parameters nicely reproduced the conformational features of RNV66 and that the presence of LNA residues significantly increased dynamic stability.

Plashkevych et al. has determined the molecular structures and dynamics of the conformationally constrained 1',2'-azetidine- and 1',2'-oxetane-fused thymidines, as well as their 2',4'-fused thymine (T) counterparts, such as LNA-T, 2'-amino LNA-T, ENA-T, and aza-ENA-T, using NMR, Ab-initio (HF/6-31G** and B3LYP/6-31++G**), and MD simulations [197]. It is discovered that these modifications fall into two different categories based on whether they result in a tricyclic 2',4'-fused system or a bicyclic 1',2'-fused system. These categories are distinguished by the internal dynamics of the glycosidic and backbone torsions, as well as by the characteristic *North-East* type sugar conformation of the 1',2'-fused systems and pure *North-type* for the 2',4'-fused nucleosides. Despite the general similarities of the *North-type* conformational constraints imposed by the 1',2'- or 2',4'-linked alteration, each group has distinct conformational spaces accessible. The nature of the sugar conformational constraints steers the physicochemical property (pK_a) of the constituent 1-thyminyl moiety, which can then be used to tune the strength of hydrogen bonding in base pairing, according to a comparison of the pK_a's of the 1-thyminyl aglycon and that of endocyclic sugar-nitrogen obtained by theoretical and experimental measurements. In due course of time newer BNA molecules will be available soon, which will give oligonucleotide-based fields of study and applications a lot of promise as antisense therapeutics.

1.5. Summary

With the ongoing progress in technology and our growing comprehension of the complexities of gene expression, ASOs have the potential to open up new avenues for personalized medicine and enable the development of novel treatments based on individual genetic profiles. The concept of using ASOs complementary to an mRNA to limit translation appears to be straightforward, but as already discussed its practical implementation still requires solving a handful of issues as development of chemically modified nucleotides with better qualities like reduced hepato-toxicity, increased target affinity and serum stability. On the good side, rapid development of methods and applications both experimentally and theoretically has resulted in creation of ASO analogues better suited to accomplish particular goals in every situation. As research continues to unravel the complexities of RNA biology, ASO therapeutics hold exciting possibilities for continued evolution and refinement.

Throughout the decade long research, ASOs have found more and more uses in fundamental science, clinics, and other environments. Applications that are specifically linked to human health include the identification and diagnosis of diseases brought on by mutations, the silencing of unwanted genes that are accountable for a variety of genetic disorders, and the manipulation of bacterial or viral genes to combat infection. There have been several attempts to lower the unfavourable expression of genes using different techniques, most of which involve the use of ASOs. The increasing use of these ASOs necessitates ongoing efforts to enhance their characteristics. The stability, specificity, and bioavailability of ASOs are the main factors that improve the utility, effectiveness and efficiency of the original nucleotide.

Over the past few years, a large number of customized ASOs has been developed. Majority of them stabilize the ASO/mRNA duplex and show improved resistance against nucleolytic breakdown. A strong antisense potency along with low toxicity has been noted in a number of newer generations ASOs that have already been employed successfully *in vivo*. The success of these ASOs encouraged researchers to look for further novel and different nucleotide analogues that would improve the oligomers' specificity, affinity, activity, and resistance to nucleases. For target validation and therapeutic applications, it is reasonable to anticipate that current developments in nucleotide chemistry will soon result in a major advancement in antisense technology.

Locked nucleic acids (LNA) are the first-generation Bridged nucleic acid (BNA) analogues combined with RNA, DNA, or other analogues to create ASOs however, their stability, bioavailability, toxicity, and, most significantly, efficiency can yet be improved. The development of more suitable analogues to improve efficiency and accomplish certain goals in each situation resulted in BNA^{NC} (2′-O,4′-aminoethylene bridged nucleic acid), a second-generation BNA analogue which demonstrated lower toxicity and, in certain situations, higher specificity in comparison to ASOs containing LNA. Nucleotides containing ribose moiety alterations have a significant intrinsic drawback in that they cannot effectively initiate RNase H cleavage of the target RNA. Therefore, gapmers that contain a segment of unmodified or phosphorothioate DNA monomers in the middle of the ON are frequently employed.

BNAs have been treated by conjugating the compounds to permeabilizing peptides, enclosing them in liposomes, employing transfecting agents, and combining them with nanoparticles. While research on BNA^{NC}-ASOs is still in its infancy, the encouraging outcomes demand greater work to include these analogues for other purposes. Promising alternatives to continue developing new and enhanced applications of oligonucleotides for research, diagnostics, and treatments are provided by the success encountered with these BNA analogues and the intense efforts to continue producing next-generation versions. However, detailed information on the various existing BNA analogues is very much necessary, which is limited indeed.

One can better understand the structural and functional significance of the BNA antisense modifications, including their higher binding affinity and increased hepatotoxicity, mechanism of action, and guidance in postulating superior antisense derivatives, by interpreting the underlying chemistry of these molecules. There have been reports of quantum chemistry research on a few antisense modifications, such as cyclohexyl PNA, MOE, and LNA, but limited study on the BNA^{NC} antisense alterations.

This thesis thus focuses on the BNA antisense alterations at the molecular and oligomer level. Since PSs have the ability to activate human RNase H, all alterations were carried out using the PS backbone linkage. By conducting a thorough DFT and MD simulation study on BNA-contained ASO/RNA duplex gapmers of varied length as 14-mer and 20-mer oligomer hybrid duplex in complex with RNase H, the oligomer hybrid duplex stability and gene silencing capability of the BNA ASOs are presented.

1.6. Objectives of the Thesis Work

This thesis is divided into seven chapters, four (chapter 3-5) of which summarizes the research findings. The conclusion and future prospective of the study are addressed separately in the end of the thesis in chapter seven. The research gap and accordingly objectives of the thesis has been summarized below as:

Research Gap: Limited computational study on existing antisense modifications**.**

- **Objective 1:** To study the structural and electronic properties of existing antisense modifications at the monomer level using DFT method, followed by development of AMBER simulation parameters and to use them for MD simulation studies at the oligomer level.

Research Gap: A combined DFT and MD based protocol for designing novel antisense modifications.

- **Objective 2:** Try to propose novel antisense modifications which may be useful to pharma industries, and to study their structural and electronic properties at the monomer level using DFT method. Next, to develop AMBER simulation parameters and use them for MD simulation studies at the oligomer level.

Research Gap: Complete mechanism of RNase H antisense activity is still unknown till date.

- **Objective 3:** To study the structure and dynamics of wild type DNA/DNA, RNA/DNA and RNA/RNA duplexes in complex with Human RNaseH (hydrolytic enzyme) catalytic domain using MD simulation studies.
- **Objective 4:** To study the structure and dynamics of the various antisense oligonucleotides of the ASO/RNA hybrid gapmer type duplexes (both existing and proposed novel antisense modifications) in complex with RNase H (hydrolytic enzyme) catalytic domain using MD simulation studies.

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